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Comparative proteomics reveals recruitment patterns of some protein families in the venoms of Cnidaria

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1   **Comparative proteomics reveals recruitment patterns of some protein families in**

2   **the venoms of Cnidaria**

3

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29

30 **Abstract**

31   Cnidarians are probably the oldest group of animals to be venomous, yet our

32 current picture of cnidarian venom evolution is highly imbalanced due to limited taxon

33 sampling. High-throughput tandem mass spectrometry was used to determine venom

34 composition of the scyphozoan *Chrysaora lactea* and two cubozoans *Tamoya*

35 *haplonema* and *Chiropsalmus quadrumanus*. Protein recruitment patterns were then

36 compared against 5 other cnidarian venom proteomes taken from the literature. A total

37 of 28 putative toxin protein families were identified, many for the first time in Cnidaria.

38 Character mapping analysis revealed that 17 toxin protein families with predominantly

39 cytolytic biological activities were likely recruited into the cnidarian venom proteome

40 before the lineage split between Anthozoa and Medusozoa. Thereafter, venoms of

41 Medusozoa and Anthozoa differed during subsequent divergence of cnidarian classes.

42 Recruitment and loss of toxin protein families did not correlate with accepted

43 phylogenetic patterns of Cnidaria. Selective pressures that drive toxin diversification

44 independent of taxonomic positioning have yet to be identified in Cnidaria and now

45 warrant experimental consideration.

46

48

Introduction

Cnidaria is believed to be the most basal of the extant Metazoa to be venomous, having evolved since Neoproterozoic times, ~650 million years ago, long before the Cambrian radiation (Van Iten *et al*., 2014). Cnidaria is a diverse phylum comprising over 13,500 free living or parasitic marine, freshwater and terrestrial species (Daly *et al*., 2007 plus myxozoans by Okamura *et al*., 2015a). Cnidaria has two major subphyla: Anthozoa and Medusozoa. Anthozoa include sea anemones and both hard and soft corals (Bridge *et al*., 1992; Marques & Collins, 2004). Medusozoa comprise the classes Staurozoa (e.g. stalked jellyfish), Cubozoa (e.g. box jellyfish), Scyphozoa (e.g. 'true' jellyfish) and Hydrozoa (e.g. *Hydra* and relatives including several species of smaller jellyfish) (Marques & Collins, 2004; Collins *et al*., 2006; Van Iten *et al*., 2014). Recent molecular phylogenetic analyses have corroborated the cnidarian nature of Myxozoa, with strong support as a sister-group to Medusozoa (reviewed in Okamura *et al*. 2015b).

The most evident synapomorphy of Cnidaria is the presence of cnidae, organelles produced by the Golgi apparatus of specialised cells called cnidoblasts (Marques & Collins, 2004; Fautin, 2009; Beckmann & Özbek, 2012). Cnidae are found in various parts of the body of a cnidarian and are classified into three morphological types: nematocysts, spirocysts and ptychocysts (Östman, 2000; Özbek *et al*., 2009). The nematocysts discharge venom and are found in all cnidarians, but are morphologically and functionally heterogeneous (David *et al*., 2008; Fautin, 2009). In addition to prey capture and defence against predation, the venom of nematocysts may also mediate spatial intraspecific and interspecific competition (Bigger, 1980; Kass-Simon & Scappaticci, 2002).

There has been resurgence in interest surrounding the nature and evolutionary origins of cnidarian venom toxins, since the first application of high throughput tandem

74   mass spectrometry realised high sequence homology between cnidarian toxins and those

75   of other venomous animals (Weston *et al*., 2012, 2013). Many studies using genomic,

76   transcriptomic or proteomic approaches have also realised these astonishing similarities

77   (Balasubramanian *et al*., 2012; Brinkman *et al*., 2012, 2015; Li *et al*., 2012, 2014, 2016;

78   Gacesa *et al*., 2015; Jouiaei *et al*., 2015a; Macrander *et al*., 2015, 2016; Lewis *et al*.,

79   2016; Ponce *et al*., 2016, Huang *et al*., 2016), leading to the recognition that

80   understanding the mechanisms underpinning toxin diversification in Cnidaria could

81   provide a platform from which the evolution of this trait in higher animals might be

82   more fully explored (Starcevic & Long, 2013; Starcevic *et al*., 2015; Jouiaei *et al*.,

83   2015b). For this to be achieved, a comprehensive inventory of toxins must first be

84   undertaken and then mapped against different taxonomic levels from established

85   cnidarian phylogeny. To date, studies attempting to infer evolutionary aspects of toxin

86   recruitment in Cnidaria have suffered limited taxon sampling, but when taken together

87   these studies have demonstrated a degree of functional recruitment of certain toxin

88   protein families at different taxonomic levels (Rachamim *et al*., 2014; Brinkman *et al*.,

89   2015; Jouiaei *et al*., 2015b). Here, the number of venom proteomes is expanded and

90   used with data from the literature for character mapping analysis, to establish a more

91   complete venom assembly hypothesis between the major taxonomic classes of Cnidaria.

92

93 **Material & Methods**

94 **Nematocyst proteomics:** The scyphozoan *Chrysaora lactea* and two cubozoans

95 *Tamoya haplonema* and *Chiropsalmus quadrumanus* (Figure 1) were collected with

96 permission (SISBIO license 15031-2) on May 7th 2012 by bottom shrimp trawls (2 cm

97 mesh size) dragged at 10 m depth along Enseada beach (Guarujá County, São Paulo

98 State, ca. 23°43'20"S 43°23'40W). Animals were identified based on morphological

99 characters (Morandini *et al.*, 2005; Morandini & Marques, 2010; Collins *et al.*, 2011)

100 and intact nematocysts were isolated from excised tentacles as previously described

101 (Weston *et al.*, 2013). To extract solubilised proteins, 1 mL of protein extraction buffer

102 (50 mM TEAB, 0.04 % (w/v) SDS, Roche protease and phosphatase inhibitor cocktail)

103 was added to freeze dried nematocysts. The reconstituted material was disrupted in a

104 sonic bath (VWR, Lutterworth, UK) for 15 mins. The debris was removed by

105 centrifugation (10,000 x g for 10 mins at 4 °C). The supernatant was decanted and the

106 soluble protein concentration determined by Bradford assay. A volume equivalent to 15

107 µg of protein was made up to 15 µL in extraction buffer and added to 15 µL 2 x

108 Laemmli sample buffer, heated for 10 mins at 95 °C and loaded onto a 4-12 % (w/v)

109 NuPAGE gel (Life Technologies) and separated by 1D SDS-PAGE. Electrophoresis was

110 performed in MES buffer (Life technologies) at 150 V for approximately 100 mins. The

111 entire gel lane was then divided into 15 equal sections, excised and cut into 2 mm

112 pieces. In-gel reduction, alkylation, and proteolytic digestion with trypsin were

113 performed as follows: Cysteine residues were reduced with 10 mM dithiothreitol and

114 alkylated with 55 mM iodoacetamide in 100 mM ammonium bicarbonate to form stable

115 carbamidomethyl derivatives. Trypsin digestion was carried out overnight at 37 °C in 50

116 mM ammonium bicarbonate buffer and the supernatant was retained. Peptides were

117 extracted from the gel pieces by two washes with 50 mM ammonium bicarbonate and

118   acetonitrile. Each wash involved shaking the gel pieces for 10 mins. The extracts were

119   pooled with the initial digestion supernatant and then lyophilised. Lyophilised extract

120   was reconstituted in 30 µL of 50 mM ammonium bicarbonate buffer for LC-MS/MS.

121

122   **Data analysis:** Data analysis was performed as previously described (Weston *et al.*,

123   2013; Gacesa *et al.*, 2015) but with minor modifications. Briefly, a one search matching

124   strategy of rawfile MS/MS data against the Tox-Prot UniProtKB/Swiss-Prot database

125   (Jungo *et al.*, 2012) using the MASCOT search engine was first executed (Perkins *et al.*,

126   1999). Methionine oxidation, phosphorylation on S/T/Y, deamidation on N/D and

127   carbamidomethyl cysteine were selected as fixed modifications. Digestion with trypsin

128   allowed up to three missed cleavages. The data were searched with a parent ion

129   tolerance of 5 ppm and a fragment ion tolerance of 0.5 Da. The MASCOT result files

130   were next uploaded into Scaffold v4.3.4 (Proteome Software, Portland, Oregon, USA)

131   (Searle, 2010) and spectra corresponding to likely venom toxin peptides were manually

132   validated for unbroken series of overlapping b-type and y-type sequence specific

133   fragments ions, where losses consistent with the sequence were acceptable. Validated

134   spectra (Figures S1-S3) corresponding to peptides with predicted venom toxin functions

135   were next distinguished from peptides with likely other non-toxic physiological

136   functions using 'ToxClassifier' (Gacesa *et al.*, 2016). This is a suite of machine learning

137   based classifiers that provide consistent discrimination of toxins from non-toxin peptide

138   sequences with > 99 % accuracy by performing BLAST and HMM comparisons against

139   the Tox-Prot UniProtKB/Swiss-Prot (Jungo *et al.*, 2012), UniProt Trembl (The UniProt

140   consortium, 2017) and NR (NCBI Resource Coordinators, 2016) databases.

141   **Character mapping analysis:** In addition to the data acquired in this study, putative

142   toxins from other cnidarians described in the literature were also included to enhance

143   the dataset. These putative toxins were from the anthozoans *Anemonia viridis*

144   (Rachamim *et al*., 2014) and *Acropora digitifera* (Gacesa *et al*., 2015), the hydrozoans

145   *Olindias sambaquiensis* (Weston *et al*., 2013) and *Hydra magnipapillata* (Rachamim *et*

146   *al*., 2014) and, the scyphozoan *Aurelia aurita* (Rachamim *et al*., 2014). The putative

147   toxins from the combined data set were assigned to venom toxin protein families using

148   established KEGG ontology. Data were coded in a matrix as presence (1) or absence (0)

149   of each toxin protein family in each species. Reconstruction of ancestral states at

150   different nodes on an accepted taxonomic tree of Cnidaria (Marques & Collins, 2004;

151   Collins *et al*., 2006) was performed using Mesquite version 3.04 (Maddison &

152   Maddison, 2015) with the parsimony criterion for the model unordered. In addition, the

153   matrix of presences and absences of toxin protein families was used to infer a

154   phylogenetic pattern based on the parsimony criterion.

155

156   **Results**

157   **Comparative proteomics of toxin protein families:** The putative toxin proteomes of

158   nematocysts for the 3 species experimentally acquired in this study are given in Table 1.

159   The toxin protein families from 5 species taken from the literature are given in Table S1.

160   A total of 28 toxin protein families were identified from the nematocyst proteomes of

161   the 8 species studied and are shown in Figure 2. Nine (~33 %) out of the 28 toxin

162   protein families were shared by all the four classes of cnidarians. These 9 protein

163   families were conotoxins O, CRISP, latrotoxin, lipase, metalloproteinase,

164   phospholipases A_2 (PLA$_2$), phospholipases D, CS αβ potassium channel blocker, and CS

165   αβ sodium channel inhibitor. Nineteen protein toxin families were not distributed across

166   all classes (Figure 2). These included three families of pore forming toxins, which were

167   the jellyfish toxin family-like proteins (JFTs) found to be restricted to the sister classes

168   Cubozoa and Scyphozoa; the actinoporins found in the classes Anthozoa, Hydrozoa and

169   Cubozoa, and latarcins found in the classes Anthozoa and Scyphozoa. The ficolins and

170   snaclec belong to the lectin families of toxins and were limited to the Scyphozoa

171   Anthozoa, and Hydrozoa. Peptides with similarity to three families of neurotoxins were

172   also taxonomically restricted (Figure 2), these were the kunitz type family detected in

173   Anthozoa and Scyphozoa, the calcium channel blocker Huwentoxin-1 reported here for

174   the first time but solely in medusozoans, and snake three finger found in all classes

175   except Cubozoa. Likewise, peptidase S1, flavin amino-oxidase and glycosyl hydrolase

176   56 families were identified in all classes except Cubozoa. Complement C3 family-like

177   proteins were identified in the Hydrozoa and Scyphozoa. MAC-PF family-like proteins

178   were identified in the Hydrozoa and Cubozoa. The presence of translationally controlled

179   tumour like proteins (TCTP) was identified in the venom proteome from both Anthozoa

180   and Hydrozoa.

181   **Evolution of the cnidarian venom arsenal:** Recruitment patterns of putative toxin

182   protein families (Figure 3 and Table S3) were inferred using a presence and absence

183   matrix (Table S2). This recruitment pattern indicated that venom of Medusozoa and

184   Anthozoa ancestors might have been composed of at least seventeen types of protein

185   toxin families (Figure 3 and Figure S4i). After separation of the ancestral lineage into

186   Anthozoa and Medusozoa, some putative toxins were lost (or not expressed) in some

187   clades. For example, the TCTP family was not present in the Cubozoa and Scyphozoa.

188   Similarly, the actinoporin toxin protein family was lost from Scyphozoa. Unlike the

189   other classes, the species of Cubozoa examined demonstrated large losses. Nine toxin

190   protein families might have been recruited by a single clade after the split Anthozoa-

191   Medusozoa (Figure 3 and Figure S4ii). Three families of cytolytic toxins (MAC-PF,

192   ficolin lectin and JFTs) appear to have been recruited into Medusozoa after the basal

193   diversification event into the venom of Hydrozoa, Scyphozoa, and Cubozoa (Figure 3).

194   Equally, two families of neurotoxins ShK-like potassium channel and sea anemone

195   sodium channel modulator appear to have been recruited into Anthozoa only. The

196   latarcin and kunitz-type toxin protein families, might have been recruited independently

197   (i.e., by convergence) into the venom of Anthozoa/Scyphozoa (Figure 3 and Figure

198   S4iii). Phylogenetic analysis of the presence and absence matrix gave a topology of

199   (Cubozoa(Anthozoa(Hydrozoa,Scyphozoa))), which disagreed with the more accepted

200   phylogeny of Cnidaria (Anthozoa(Hydrozoa(Cubozoa,Scyphozoa))).

201
202   **Discussion**

203   The putative toxin component of nematocyst proteomes for 3 out of the 8 species

204   examined (*Chrysaora lactea, Tamoya haplonema*, and *Chiropsalmus quadrumanus*) are

205   described in this study for the first time (Table 1). Venom data from 3 other species

206 (*Anemonia viridis*, *Hydra magnipapillata*, and *Aurelia aurita*) were published elsewhere

207 (Rachamim *et al.*, 2014) and reassessed in this study. These data were combined with

208 our own previously published putative nematocyst toxin proteomes from *Acropora*

209 *digitifera* (Gacesa *et al.*, 2015) and *Olindias sambaquiensis* (Weston *et al.*, 2013).

210 Altogether, the data from this study has supported previous research that Anthozoa and

211 Medusozoa have complex venom composition comprising multiple protein families

212 (Rachamim *et al.*, 2014; Jouiaei *et al.*, 2015c) (Figures 2 and 3). We highlight that,

213 although transcriptomes and proteomes from other species of cnidarians have also been

214 published (Moran *et al.*, 2013; Jouiaei *et al.*, 2015a, Ponce *et al.*, 2016, Macrander *et*

215 *al.*, 2016), our analysis focused on those species for which we had access to raw

216 proteomics MS/MS data which could be analysed using identical bioinformatics

217 methods, ensuring results were fully comparable. Our study was conservative, being

218 restricted to putative toxin annotation in the expressed proteome and did not include a

219 study of transcriptomes. This was because not all the transcripts that contributed to

220 transcriptome diversity would equally be likely to be translated (if at all) and have

221 contributed to protein diversity. Hence, correlation between sequences annotated as

222 putative toxins in the transcriptome and proteome would not have been straightforward

223 given the difficulty in differentiating sequences with toxic and other physiological

224 functions. Future work to overcome this impediment will require the acquisition of

225 genome sequence onto which other sequence data can be mapped (Gacesa *et al.*, 2015).

226

227 **Comparative venom proteomic analysis from different Cnidaria classes**

228   Our comparative proteomics data of putative venom toxins indicated that nearly

229 half of the protein toxin families were distributed across all of the cnidarian classes

230 studied (Figure 2). The biological activities of some of these toxin families are of note,

231   for example, PLA$_2$ toxins have thus far only been identified with haemolytic activity in

232   cnidarians (Hessinger & Lenhoff, 1976; Grotendorst & Hessinger, 2000; Anderluh &

233   Maček, 2002; Talvinen & Nevalainen, 2002; Nevalainen *et al*., 2004; Razpotnik *et al*.,

234   2010), although neurotoxic and myotoxic activities as well as non-toxic physiological

235   functions have also been widely reported in other venomous animals (Fry *et al*., 2009;

236   Six & Dennis, 2000). Likewise, phospholipase D family proteins isolated from

237   cnidarian venoms have been reported to exhibit necrotic activity (Burke, 2002; Uri *et*

238   *al*., 2005), with homologs also recently identified in the giant jellyfish *Cyanea capillata*

239   (Liu *et al*., 2015). Most of the metalloproteinases identified in this study belonged to the

240   zinc metalloproteinase family. This family of toxins is an important component found in

241   the venoms of many terrestrial animals such as centipedes, snakes and ticks (Fry *et al.*,

242   2009; Undheim *et al*., 2014), with diverse biological activities culminating in

243   haemorrhage and tissue necrosis in the target prey following envenomation (Fox &

244   Serrano, 2005; da Silveira *et al*., 2007). Transcriptomic and proteomic studies have

245   identified zinc metalloprotease in venoms of the scyphozoans *Stomolophus meleagris*,

246   *Cyanea capillata*, and *Cyanea nozakii* (Li *et al*., 2014, 2016; Liu *et al*., 2015), the

247   cubozoan *Chironex fleckeri* (Brinkman *et al*., 2015; Jouiaei *et al*., 2015a) and the

248   anthozoan *Anthopleura elegantissima* (Macrander *et al*., 2015). A study of

249   metalloproteases from the scyphozoan *Nemopilema nomurai*, *Rhopilema esculenta*,

250   *Cyanea nozakii*, and *Aurelia aurita* confirmed the necrotic toxicity of these enzymes

251   (Lee *et al*., 2011). Both sodium and potassium ion channel inhibitors were identified in

252   representatives of all of the classes examined. These two types of neurotoxins have been

253   widely studied in Anthozoa, especially sea anemones and comprise the largest number

254   of toxins so far recorded in public databases for Cnidaria (Moran *et al*., 2009; Šuput,

255   2009; Turk & Kem, 2009; Frazão *et al*., 2012; Jouiaei *et al*., 2015c; Macrander *et al*.,

256 2015; Mariottini *et al*., 2015). Neurotoxic effects have been identified in scyphozoans

257 such as *Cyanea nozakii* (Feng *et al*., 2010), *Cyanea capillata* (Helmholz *et al*., 2012),

258 and *Pelagia noctiluca* (Pang *et al*., 1993; Morabito *et al*., 2012) and, in cubozoans such

259 as *Carukia barnesi* (Winkel *et al*., 2005) and *Malo kingi* (Gershwin, 2007). In this study,

260 we identified two putative types of sodium and potassium ion channel inhibitors

261 (Figure 2, Table S2). ShK-like potassium channel and sea anemone sodium channel

262 modulator were only found in a single Anthozoan species, *Anemonia viridis* (a sea

263 anemone). It should be noted that this was the only species of sea anemone analysed in

264 this study. Both sodium and potassium putative ion channel inhibitors have been found

265 exclusively in sea anemones (Moran *et al*., 2009; Diochot and Lazdunski, 2009). CS αβ

266 potassium channel blocker and CS αβ sodium channel inhibitor were found in all of the

267 species of cnidarians analysed including another anthozoan, *Acropora digitifera* and

268 have sequence homology to sodium and potassium channel blockers of scorpions. This

269 observation might highlight a rare example of mechanistic convergence whereby

270 sodium and potassium ion channel inhibitors appeared on two separate occasions within

271 the cnidarians. Convergent evolution of these toxins in scorpions and sea anemones has

272 been previously reported and although these toxin protein families are structurally

273 different, functional mapping studies have shown similarities in the binding cores

274 (Gasparini*et al*., 2004). Further species sampling is required to substitute these

275 observations which are based here on a single MS/MS event in the anthozoa *Acropora*

276 *digitifera,* the hydrozoa *Hydra magnipapillata*, the scyphozoan *Aurelia aurita* and the

277 cubozoans *Chiropsalmus quadrumanus* and *Tamoya haplonema*. It should also be noted

278 that the names given to each putative ion channel inhibitors were used to distinguish

279 between the two different possible origins of the putative sodium and potassium ion

280 channel inhibitors identified in this study. Another family of neurotoxins were the

281 CRISP type toxins, which again were found in all classes of cnidarians studied herein.

282 This toxin protein family has widely been reported in cnidarian venoms (Brinkman *et*

283 *al.*, 2015; Ponce *et al.*, 2016; Lewis *et al.*, 2016) and attributed many biological

284 functions.

285   Just over half of the toxins protein families identified in this study were

286 restricted to certain cnidarian classes only (Figure 2). Hyaluronidase-like proteins were

287 found in all classes of cnidarians except Cubozoa, but these proteins are common and

288 have non-toxic physiological function in many non-venomous animals. It is feasible that

289 such proteins are likely recruited into venoms not as toxins, but as adjuvants to increase

290 tissue permeability (Kemparaju & Girish, 2006; Fry *et al.*, 2009). Non-toxic peptides

291 and proteins present in nematocysts that may function in toxin maturation, toxin

292 trafficking and delivery, or as self-defence mechanisms against the biological activities

293 of the venom have received little study and perhaps warrant closer inspection. Likewise,

294 the peptidase S1 family was also detected in all cnidarian classes studied except

295 Cubozoa. This family is part of the group of serine protease inhibitors that is widely

296 distributed in other marine venomous animals including marine cone snails and

297 cephalopods (Mourão & Schwartz, 2013), as well as terrestrial reptiles (Fry e*t al*.,

298 2009). Recently, serine protease homologs were identified in the transcriptome of the

299 sea anemone *Anthopleura elegantissima* (Macrander *et al.*, 2015). However, the

300 biological activity of the S1 peptidase family of toxins has yet to be confirmed in

301 cnidarians. It is unclear why proteins commonly associated with innate immune

302 responses are also apparently widely distributed in cnidarian venoms. For example,

303 MAC-PF-like toxins have also been identified in sea anemones (Nagai *et al.*, 2002b;

304 Oshiro *et al.*, 2004) and were recently annotated in the transcriptomes and proteomes of

305 Hydrozoa and Scyphozoa (Rachamim *et al.*, 2014). Likewise, the actinoporins are pore-

306 forming toxins were found in the proteomes of Anthozoa and Cubozoa classes. These

307 cytolysins have also been identified in transcriptome sequences and biological activity

308 recorded in nematocyst venom extracts of various *Hydra* species (Hydrozoa) (Hwang *et*

309 *al*., 2007; Glasser *et al*., 2014).

310

Assembly of the cnidarian venom proteome

312 To date, only one previous study has been published that used similar

313 approaches to those described here to investigate evolutionary aspects of toxin

314 recruitment in Cnidaria (Rachamim *et al*., 2014). In this previous study, the kunitz type

315 family of toxins was only found in Anthozoa. In the study present here, this family of

316 toxins was found in Anthozoa, but was also identified in Scyphozoa. In the study of

317 Rachamim *et al*., (2014), the PLA2 family of toxin proteins were only found in species

318 of Scyphozoa and Hydrozoa. In our analyses, PLA2 were present in all the Cnidaria

319 classes studied and hence, most likely arose as a recruitment event at the base ancestor

320 of the Cnidaria. It should be noted that PLA2 like proteins have also been identified in

321 recent studies of Anthozoan venoms (Macrander *et al*., 2015, 2016). Differences in the

322 recruitment patterns between studies might be explained because of the low number of

323 species sampled. The extent of comparison groups (all Cnidaria) in light of the

324 sparseness of data at terminals in the analysis is a concern, for example, no data is

325 currently available on the toxin compliment of venoms from the Staurozoa or Myxozoa

326 (Marques & Collins, 2004; Okamura *et al*., 2015a). Based on the data presented, many

327 of the neurotoxic and cytotoxic protein toxin families might have been recruited into the

328 venom proteome early in cnidarian evolution, before the first major radiation in this

329 phylum around 800 million years ago (Park *et al.*, 2012; Van Iten *et al.*, 2017).

330   The approaches used in this study were very conservative, with analyses based

331   exclusively on putative toxin protein families found in each proteomic profile and not

332   specific toxin peptides or proteins. These proteomic profiles can be considered

333   phenotypes, or a "morphological representation" of the venom, allowing variation in the

334   toxin complement to be evaluated. For example, in this study JFTs were found only in

335   the venoms of Scyphozoa and Cubozoa. However, previous studies have demonstrated

336   JFTs encoded in the genome and expressed in the proteome of Hydrozoa, and in the

337   transcriptome of the anthozoan *Anemonia viridis* (Rachamim *et al*., 2014). Few reports

338   in the literature have documented variation in toxin composition of venom at taxonomic

339   level in the phylum Cnidaria (Orts *et al*., 2013; Rachamim *et al*., 2014), and certainly

340   there have been no studies that have attempted to put into context what the biological

341   consequences of venom variation might be (Gacesa *et al*., 2015; Knittell *et al*., 2016).

342   The difference between the two phylogenetic patterns (accepted vs inferred using the

343   presence and absence matrix) found in this study could be due to various ecological

344   factors that need to be investigated in future studies. But increased sampling and

345   analysis at different taxonomic levels is a priority in order to identify the influence of

346   history and ecology in the origin of these contrasting patterns.

347

348   **Conclusions**

349   Venom composition of Medusozoa and Anthozoa are different, with cytolytic

350   toxin protein families slightly more abundant in Medusozoa. When only toxin protein

351   family composition was used for phylogenetic inference, the resulting topology

352   (Cubozoa(Anthozoa(Hydrozoa,Scyphozoa))) did not match the classic published

353   phylogeny (Anthozoa(Hydrozoa(Cubozoa,Scyphozoa))). Understanding the functional

354   context (environment versus morphological form) that may drive expression of toxins in

355 Cnidaria requires future experimentally consideration, including wider taxonomic

356 sampling.

357

373

374 **References**

375 Anderluh G, Maček, P. 2002. Cytolytic peptide and protein toxins from sea anemones

376 (Anthozoa: Actiniaria). Toxicon 40:111–124 DOI:10.1016/S0041-0101(01)00191-X.

377   Balasubramanian PG, Beckmann A, Warnken U, Schnölzer M, Schüler A, Bornberg-

378   Bauer E, Holstein TW, Özbek S. 2012. Proteome of *Hydra* nematocyst. The Journal of

379   Biological Chemistry 287:9672–9681 DOI: 10.1074/jbc.M111.328203.

380   Beckmann A, Özbek S. 2012. The nematocyst: A molecular map of the cnidarian

381   stinging organelle. The International Journal of Developmental Biology 56:577–582

382   DOI: 10.1387/ijdb.113472ab.

383   Bigger CH. 1980. Interspecific and intraspecific acrorhagial aggressive behaviour

384   among sea anemones: a recognition of self and not self. The Biological Bulletin

385   159:117–134 DOI: 10.2307/1541013.

386   Bridge D, Cunningham CW, DeSalle R, Buss LW. 1992. Class-level relationships in the

387   phylum Cnidaria: Evidence from mitochondrial genome structure. Proceedings of the

388   National Academy of Sciences of the United States of America 89:8750–8753

389   DOI:10.1073/pnas.89.18.8750.

390   Brinkman DL, Aziz A, Loukas A, Potriquet J, Seymour J, Mulvenna J. 2012. Venom

391   proteome of the box jellyfish *Chironex fleckeri* PLoS ONE 7: e47866

392   DOI:10.1371/journal.pone.0047866.

393   Brinkman DL, Jia X, Potriquet J, Kumar D, Dash D, Kvaskoff D, Mulvenna J. 2015.

394   Transcriptome and venom proteome of the box jellyfish *Chironex fleckeri*. BMC

395   Genomics 16 DOI: 10.1186/s12864-015-1568-3

396   Burke WA. Cnidarians and human skin. 2002. Dermatologic Therapy 15:18–25 DOI:

397   10.1046/j.1529-8019.2002.01508.x.

398 Collins AG, Bentlage B, Gillan W, Lynn TH, Morandini AC, Marques AC. 2011.

399 Naming the Bonaire banded box jelly, *Tamoya ohboya*, n. sp. (Cnidaria: Cubozoa:

400 Carybdeida: Tamoyidae). Zootaxa 2753:53–68 DOI: http://dx.doi.org/10.11646/%25x

401 Collins AG, Schuchert P, Marques AC, Jankowski T, Medina M, Schierwater B. 2006.

402 Medusozoan phylogeny and character evolution clarified by new large and small

403 subunit rDNA data and an assessment of the utility of phylogenetic mixture models.

404 Systematic Biology 55:97–115 DOI: 10.1080/10635150500433615

405 Da Silveira RB, Wille ACM, Chaim OM, Appel MH, Silva DT, Franco CRC, Toma L,

406 Mangili OC, Gremski W, Dietrich CP, Nader HB, Veiga SS. 2007. Identification,

407 cloning, expression and functional characterization of an astacin-like metalloprotease

408 toxin from *Loxosceles intermedia* (Brown spider) venom. Biochemical Journal

409 406:355–363 DOI: 10.1042/BJ20070363.

410 Daly M, Brugler MR, Cartwright P, Collins AG, Dawson MN, Fautin DG, France SC,

411 McFadden CS, Opresko DM, Rodriguez E, Romano SL, Stake JL. 2007. The phylum

412 Cnidaria: A review of phylogenetic patterns and diversity 300 years after Linnaeus.

413 Zootaxa 1668:127–182.

414 David CN, Özbek S, Adamczyk P, Meier S, Pauly B, Chapman J, Hwang JS, Gojobori

415 T, Holstein TW. 2008. Evolution of complex structures: minicollagens shape the

416 cnidarian   nematocyst.   Trends   in   Genetics.   24:431–438   DOI:

417 http://dx.doi.org/10.1016/j.tig.2008.07.001.

418 Diochot S, Lazdunski M. 2009. Sea anemone toxins affecting potassium channels. In:

419 Fusetani N, Kem W, eds. Marine toxins as research tools. Cham: Springer, 99–122.

420 Fautin DG. 2009. Structural diversity, systematics, and evolution of cnidae. Toxicon

421 54:1054–1064 DOI:10.1016/j.toxicon.2009.02.024

422 Feng J, Yu H, Xing R, Liu S, Wang L, Cai S, Li P. 2010. Isolation and characterization

423 of lethal proteins in nematocyst venom of the jellyfish *Cyanea nozakii Kishinouye*.

424 Toxicon 55:118–125 DOI:10.1016/j.toxicon.2009.07.008

425 Fox JW, Serrano SMT. 2005. Structural considerations of the snake venom

426 metalloproteinases, key members of the M12 reprolysin family of metalloproteinases.

427 Toxicon 45: 969–985 DOI:10.1016/j.toxicon.2005.02.012.

428 Frazão B, Vasconcelos V, Antunes A. Sea anemone (Cnidaria, Anthozoa, Actiniaria)

429 toxins: An overview. 2012. Marine Drugs 10:1812–1851 DOI:10.3390/md10081812.

430 Fry BG, Roelants K, Champagne DE, Scheib H, Tyndall JD, King GF, Nevalainen TJ,

431 Norman J, Lewis RJ, Norton RS, Renjifo C, de la Vega RCR. 2009. The toxicogenomic

432 multiverse: convergent recruitment of proteins into animal venoms. Annual Reviews of

433 Genomics and Human Genetics 10:483–511 DOI:

434 10.1146/annurev.genom.9.081307.164356.

435 Gacesa R, Chung R, Dunn SR, Weston A, Jaimes-Becerra A, Marques AC, Morandini

436 A, Hranueli D, Starcevic A, Ward M, Long PF. 2015. Gene duplications are extensive

437 and contribute significantly to the toxic proteome of nematocysts isolated from

438 *Acropora digitifera* (Cnidaria: Anthozoa: Scleractinia). BMC Genomics 16:774 DOI:

439 10.1186/s12864-015-1976-4.

440 Gacesa R, Barlow D, Long P. 2016. Machine learning can differentiate venom toxins

441 from other proteins having non-toxic physiological functions. PeerJ Computer Science

442 2:e90 DOI 10.7717/peerj-cs.90.

443 Gasparini S, Gilquin B, Ménez A. 2004. Comparison of sea anemone and scorpion

444 toxins binding to Kv1 channels: an example of convergent evolution. Toxicon 43:901–

445 908 DOI: 10.1016/j.toxicon.2004.03.029.

446   Gershwin LA. 2007. *Malo kingi*: A new species of Irukandji jellyfish (Cnidaria:

447   Cubozoa: Carybdeidae), possibly lethal to humans, from Queensland, Australia.

448   Zootaxa 68:55–68.

449   Glasser E, Rachamim T, Aharanovich D, Sher D. 2014. Hydra actinoporin-like toxin-1,

450   an unusual hemolysin from the nematocyst venom of Hydra magnipapillata which

451   belongs   to   an   extended   gene   family.   Toxicon   91:103–113

452   DOI:https://doi.org/10.1016/j.toxicon.2014.04.004

453   Grotendorst GR, Hessinger DA. 2000. Enzymatic characterization of the major

454   phospholipase A2 component of sea anemone (*Aiptasia pallida*) nematocyst venom.

455   Toxicon 38:931–943 DOI:10.1016/S0041-0101(99)00206-8.

456   Helmholz H, Wiebring A, Lassen S, Ruhnau C, Schuett C, Prange A. 2012. Cnidom

457   analysis combined with an *in vitro* evaluation of the lytic, cyto- and neurotoxic potential

458   of *Cyanea capillata* (Cnidaria: Scyphozoa). Scientia Marina 76: 339–348

459   http://dx.doi.org/10.3989/scimar.03381.16E.

460   Hessinger DA, Lenhoff HM. 1976. Mechanism of haemolysis induced by nematocyst

461   venom: Roles of phospholipase A and direct lytic factor. Archives of Biochemistry and

462   Biophysics 173:603–613 DOI:10.1016/0003-9861(76)90297-6.

463   Huang C. Morlighem JE. Zhou H. Lima EP. Gomes PB. Cai J. Lou I. Perez C. Lee SM.

464   Rádis-Baptista G. 2016. The Transcriptome of the zoanthid *Protopalythoa variabilis*

465   (Cnidaria, Anthozoa) predicts a basal repertoire of toxin-like and venom-auxiliary

466   polypeptides. Genome Biology and Evolution 8:3045–3064 doi:10.1093/gbe/evw204.

467   Hwang JS, Ohyanagi H, Hayakawa S, Osato N, Nishimiya-Fujisawa C, Ikeo K, David

468   C, Fujisawa T, Gojobori T. 2007. The evolutionary emergence of cell type-specific

469   genes inferred from the gene expression analysis of Hydra. Proceedings of the National

470 Academy of Sciences of the United States of America 104: 14735–14740 DOI:

471 10.1073/pnas.0703331104.

472 Jouiaei M, Casewell N, Yanagihara A, Nouwens A, Cribb B, Whitehead D, Jackson T,

473 Ali S, Wagstaff S, Koludarov I, Alewood P, Hansen J, Fry B. 2015a. Firing the sting:

474 Chemically induced discharge of cnidae reveals novel proteins and peptides from box

475 jellyfish (*Chironex fleckeri*) venom. Toxins 7:936–950 DOI:10.3390/toxins7030936.

476 Jouiaei M, Sunagar K, Gross AF, Scheib H, Alewood PF, Moran Y, Fry BG. 2015b.

477 Evolution of an ancient venom: recognition of a novel family of cnidarian toxins and

478 the common evolutionary origin of sodium and potassium neurotoxins in sea anemone.

479 Molecular Biology and Evolution 32:1598–1610 DOI: 10.1093/molbev/msv050.

480 Jouiaei M, Yanagihara AA, Madio B, Nevalainen TJ, Alewood PF, Fry BG. 2015c.

481 Ancient venom systems: A review on Cnidaria toxins. Toxins 7:2251-2271 DOI:

482 10.3390/toxins7062251.

483 Jungo F, Bougueleret L, Xenarios I, Poux S. 2012. The UniProtKB/Swiss-Prot Tox-Prot

484 program: a central hub of integrated venom protein data. Toxicon 60:551–557 DOI:

485 10.1016/j.toxicon.2012.03.010.

486 Kass-Simon G, Scappaticci J. 2002. The behavioural and developmental physiology of

487 nematocysts. Canadian Journal Zoology 80:1772–1794 DOI: 10.1139/z02-135.

488 Kemparaju K, Girish KS. 2006. Snake venom hyaluronidase: A therapeutic target. Cell

489 Biochemistry and Function 24:7–12 DOI: 10.1002/cbf.1261.

490 Knittel P, Long PF, Brammall L, Marques AC, Almeida MT, Padilla G, Moura-da-Silva

491 AM. 2016. Characterising the enzymatic profile of crude tentacle extracts from the

492 South Atlantic jellyfish *Olindias sambaquiensis* (Cnidaria: Hydrozoa). Toxicon 119:1–7

493 DOI: http://dx.doi.org/10.1016/j.toxicon.2016.04.048.

494 Lee H, Jung ES, Kang C, Yoon WD, Kim JS, Kim E. 2011. Scyphozoan jellyfish venom

495 metalloproteinases and their role in the cytotoxicity. Toxicon 58:277–284

496 DOI:10.1016/j.toxicon.2011.06.007.

497 Lewis-Ames C, Ryan J, Bely A, Cartwright P, Collins A. 2016. A new transcriptome and

498 transcriptome profiling of adult and larval tissue in the box jellyfish *Alatina alata*: an

499 emerging model for studying venom, vision and sex. BMC Genomics 17:650

500 DOI:10.1186/s12864-016-2944-3.

501 Li R, Yu H, Xing R, Liu S, Qing Y, Li K, Li B, Meng X, Cui J, Li P. 2012. Application

502 of nanoLC-MS/MS to the shotgun proteomic analysis of the nematocyst proteins from

503 jellyfish *Stomolophus meleagris*. Journal of Chromatography B 899:86–95

504 DOI:10.1016/j.jchromb.2012.05.006.

505 Li R, Yu H, Xue W, Yue Y, Liu S, Xing R, Li P. 2014. Jellyfish venomics and venom

506 gland transcriptomics analysis of *Stomolophus meleagris* to reveal the toxins associated

507 with sting. Journal of Proteomics 106:17–29 DOI:10.1016/j.jprot.2014.04.011.

508 Li R, Yu H, Yue Y, Liu S, Xing R, Chen X, Li P. 2016. Combined proteomics and

509 transcriptomics identifies sting-related toxins of Jellyfish *Cyanea nozakii*. Journal of

510 proteomics 148:57–64 DOI:http://dx.doi.org/10.1016/j.jprot.2016.07.023.

511 Liu G, Zhou Y, Liu D, Wang Q, Ruan Z, He Q, Zhang L. 2015. Global transcriptome

512 analysis of the tentacle of the jellyfish *Cyanea capillata* using deep sequencing and

513 expressed sequence tags: Insight into the toxin- and degenerative disease-related

514 transcripts. PLoS ONE 10:e0142680

515 DOI:http://dx.doi.org/10.1371/journal.pone.0142680.

516   Macrander J, Brugler MR, Daly M. 2015. A RNA-seq approach to identify putative

517   toxins from acrorhagi in aggressive and non-aggressive *Anthopleura elegantissima*

518   polyps. BMC Genomics 16:221 DOI: 10.1186/s12864-015-1417-4.

519   Macrander J, Broe M, Daly M. 2016. Tissue-specific venom composition and

520   differential gene expression in sea anemones. Genome Biology and Evolution

521   DOI:10.1093/gbe/evw155.

522   Maddison WP, Maddison DR. 2015. Mesquite: a modular system for evolutionary

523   analysis. Version 3.04 http://mesquiteproject.org.

524   Mariottini GL, Bonello G, Giacco E, Pane L. 2015. Neurotoxic and neuroactive

525   compounds from Cnidaria: Five decades of research…. and more. Central Nervous

526   System Agents in Medicinal Chemistry 15:74–80 DOI:

527   10.2174/1871524915666150309141900.

528   Marques AC, Collins AG. 2004. Cladistic analysis of Medusozoa and cnidarian

529   evolution. Invertebrate Biology 123:23–42 DOI: 10.1111/j.1744-7410.2004.tb00139.x.

530   Morabito R, Condello S, Currò M, Marino A, Ientile R, La Spada G. 2012. Oxidative

531   stress induced by crude venom from the jellyfish *Pelagia noctiluca* in neuronal-like

532   differentiated SH-SY5Y cells. Toxicology in Vitro 26:694–699

533   DOI:10.1016/j.tiv.2012.03.002.

534   Moran Y, Gordon D, Gurevitz M. 2009. Sea anemone toxins affecting voltage-gated

535   sodium channels - molecular and evolutionary features. Toxicon 54:1089–1101

536   DOI:10.1016/j.toxicon.2009.02.028

537   Moran Y, Praher D, Schlesinger A, Ayalon A, Tal Y, Technau U. 2013. Analysis of

538   soluble protein contents from the nematocysts of a model sea anemone sheds light on

539   venom evolution. Marine Biotechnology 15: 329–339 DOI: 10.1007/s10126-012-9491-

540   y.

541   Morandini AC, Ascher D, Stampar SN, Ferreira JFV. 2005. Cubozoa e Scyphozoa

542   (Cnidaria: Medusozoa) de águas costeiras do Brasil. Iheringia Série Zoologia 95:281–

543   294 DOI: http://dx.doi.org/10.1590/S0073-47212005000300008.

544   Morandini AC, Marques AC. 2010. Revision of the genus *Chrysaora* Péron & Lesueur,

545   1810 (Cnidaria: Scyphozoa). Zootaxa 2464:1–97 DOI:http://dx.doi.org/10.11646/%25x.

546   Mourão CBF, Schwartz EF. 2013. Protease inhibitors from marine venomous animals

547   and their counterparts in terrestrial venomous animals. Marine Drugs 11:2069–2112

548   DOI:10.3390/md11062069.

549   Nagai H, Oshiro N, Takuwa-Kuroda K, Iwanaga S, Nozaki M, Nakajima T. 2002. Novel

550   proteinaceous toxins from the nematocyst venom of the Okinawan sea anemone

551   *Phyllodiscus semoni* Kwietniewski. Biochemical and Biophysical Research

552   Communications 294:760–763 DOI:10.1016/S0006-291X(02)00547-8.

553   NCBI Resource Coordinators. 2016. Database resources of the National Center for

554   Biotechnology Information. Nucleic Acids Research 44:D7–D19 DOI:

555   10.1093/nar/gkv1290.

556   Nevalainen TJ, Peuravuori HJ, Quinn RJ, Llewellyn LE, Benzie JH, Fenner PJ, Winkel

557   KD. 2004. Phospholipase A2 in Cnidaria. Comparative Biochemistry and Physiology

558   Part B: Biochemistry and Molecular Biology 139:731–735

559   DOI:10.1016/j.cbpc.2004.09.006.

560 Okamura B, Gruhl A, Reft AJ. 2015a. Cnidarian origins of Myxozoa. In: Okamura B,

561 Gruhl A, Bartholomew JL, eds. Myxozoan Evolution, Ecology and Development.

562 Cham: Springer, 45–68.

563 Okamura B, Gruhl A, Bartholomew J. 2015b. An introduction to myxozoan evolution,

564 ecology and development. In: Okamura B, Gruhl A, Bartholomew JL, eds. Myxozoan

565 Evolution, Ecology and Development. Cham: Springer, 1-20.

566 Orts DJB, Peigneur S, Madio B, Cassoli JS, Montandon GG, Pimenta AMC, Bicudo

567 JEPW, Freitas JC, Zaharenko AJ, Tytgat J. 2013. Biochemical and electrophysiological

568 characterization of two sea anemone Type 1 potassium toxins from a geographically

569 distant population of *Bunodosoma caissarum*. Marine Drugs 11:655–679

570 DOI:10.3390/md11030655.

571 Oshiro N, Kobayashi C, Iwanaga S, Nozaki M, Namikoshi M, Spring J, Nagai H. 2004.

572 A new membrane-attack complex/perforin (MACPF) domain lethal toxin from the

573 nematocyst venom of the Okinawan sea anemone *Actineria villosa*. Toxicon 43:225–

574 228 DOI:10.1016/j.toxicon.2003.11.017.

575 Östman C. 2000. A guideline to nematocyst nomenclature and classification, and some

576 notes on the systematic value of nematocysts. Scientia Marina 64:31–46

577 DOI:10.3989/scimar.2000.64s131

578 Özbek S, Balasubramanian PG, Holstein TW. 2009. Cnidocyst structure and the

579 biomechanics of discharge. Toxicon 54:1038–1045 DOI:10.1016/j.toxicon.2009.03.006.

580 Pang KA, Schawrtz MS. 1993. Guillain-Barré syndrome following jellyfish stings

581 (*Pelagia noctiluca*). Journal of Neurology, Neurosurgery and Psychiatry 56:1133–1137

582 DOI:10.1136/jnnp.56.10.1133.

583 Park E, Hwang DS, Lee SJ, Song JI, Seo TK, Won YJ. 2012. Estimation of the

584 divergence times in cnidarian evolution based on mitochondrial proteincoding genes

585 and the fossil record. Molecular Phylogenetics and Evolution 62:329–345

586 DOI:10.1016/j.ympev.2011.10.008.

587 Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. 1999. Probability-based protein

588 identification by searching sequence databases using mass spectrometry data.

589 Electrophoresis 20:3551–3567 DOI: 10.1002/(SICI)1522-

590 2683(19991201)20:18<3551::AID-ELPS3551>3.0.CO;2-2.

591 Ponce D, Brinkman D, Potriquet J, Mulvenna J. 2016. Tentacle transcriptome and

592 venom proteome of the pacific sea nettle, *Chrysaora fuscescens* (Cnidaria: Scyphozoa).

593 Toxins 8:102 DOI:10.3390/toxins8040102.

594 Rachamim T, Morgenstern D, Aharonovich D, Brekhman V, Lotan T, Sher D. 2014. The

595 dynamically evolving nematocyst content of an anthozoan, a scyphozoan, and a

596 hydrozoan. Molecular Biology and Evolution 32:740–753 DOI:

597 10.1093/molbev/msu335.

598 Razpotnik A, Križaj I, Šribar J, Kordiš D, MačEk P, Frangež R, Kem WR, Turk T. 2010.

599 A new phospholipase A2 isolated from the sea anemone *Urticina crassicornis* - Its

600 primary structure and phylogenetic classification. The FEBS Journal 277:2641–2653

601 DOI: 10.1111/j.1742-4658.2010.07674.x.

602 Searle BC. 2010. Scaffold: a bioinformatic tool for validating MS/MS-based proteomic

603 studies. Proteomics 10:1265–1269 DOI: 10.1002/pmic.200900437.

604 Six DA, Dennis EA. 2000. The expanding superfamily of phospholipase A2 enzymes:

605 Classification and characterization. Biochimica et Biophysica Acta (BBA) - Molecular

606 and Cell Biology of Lipids 1488:1–19 DOI:10.1016/S1388-1981(00)00105-0.

607   Starcevic A, Long PF. 2013. Diversification of animal venom peptides-were jellyfish

608   amongst the first combinatorial chemists? ChemBioChem 14:1407–1409 DOI:

609   10.1002/cbic.201300305.

610   Starcevic A, Moura-da-Silva AM, Cullum J, Hranueli D, Long PF. 2015. Combinations

611   of long peptide sequence blocks can be used to describe toxin diversification in

612   venomous animals. Toxicon 95:84–92 DOI:10.1016/j.toxicon.2015.01.005.

613   Šuput D. 2009. *In vivo* effects of cnidarian toxins and venoms. Toxicon 54:1190–1200

614   DOI:10.1016/j.toxicon.2009.03.001.

615   Talvinen KA, Nevalainen TJ. 2002. Cloning of a novel phospholipase A2 from the

616   cnidarian *Adamsia carciniopados*. Comparative Biochemistry and Physiology Part B:

617   Biochemistry   and   Molecular   Biology.   132:571–578   DOI:10.1016/S1096-

618   4959(02)00073-8.

619   The UniProt Consortium. 2017. UniProt: the universal protein knowledgebase. Nucleic

620   Acids Research. 45:D158–D159 DOI:https://doi.org/10.1093/nar/gkw1099.

621   Turk T, Kem WR. 2009. The phylum Cnidaria and investigations of its toxins and

622   venoms until 1990. Toxicon 54:1031–1037 DOI:10.1016/j.toxicon.2009.06.031.

623   Undheim EB, Jones A, Clauser KR, Holland JW, Pineda SS, King GF, Fry BG. 2014.

624   Clawing through evolution: Toxin diversification and convergence in the ancient lineage

625   Chilopoda (centipedes). Molecular Biology and Evolution 31:2124–2148 DOI:

626   10.1093/molbev/msu162.

627   Uri S, Marina G, Liubov G. 2005. Severe delayed cutaneous reaction due to

628   Mediterranean jellyfish (*Rhopilema nomadica*) envenomation. Contact Dermatitis

629   52:282–283 DOI: 10.1111/j.0105-1873.2005.00582.x.

630 Van Iten H, Marques AC, Leme JDM, Pacheco MLF, Simões MG. 2014. Origin and

631 early diversification of the phylum Cnidaria Verrill: Major developments in the analysis

632 of the taxon's Proterozoic-Cambrian history. Palaeontology 57:677–690 DOI:

633 10.1111/pala.12116.

634 Van Iten H, Leme JM, Pacheco MLAF, Simões MG, Fairchild TR, Rodrigues F, Galante

635 D, Boggiani PC, Marques AC. 2016. Origin and early diversification of phylum

636 Cnidaria: key macrofossils from the Ediacaran system of North and South America. In:

637 The Cnidaria, past, present and future, Goffredo, S. & Dubinsky, Z. (Ed.), Springer. pp.

638 31-40 (ISBN 978-3-319-31303-0; ISBN 978-3-319-31305-4 – e-book; 10.1007/978-3-

639 319-31305-4_3).

640 Weston AJ, Chung R, Dunlap WC, Morandini AC, Marques AC, Moura-da-Silva AM,

641 Ward M, Padilla G, da Silva LF, Andreakis N, Long PF. 2013. Proteomic

642 characterisation of toxins isolated from nematocysts of the South Atlantic jellyfish

643 *Olindias sambaquiensis*. Toxicon 71:11–17 DOI:10.1016/j.toxicon.2013.05.002.

644 Weston AJ, Dunlap WC, Shick JM, Klueter A, Iglic K, Vukelic A, Starcevic A, Ward M,

645 Wells ML, Trick CG, Long PF. 2012. A profile of an endosymbiont-enriched fraction of

646 the coral *Stylophora pistillata* reveals proteins relevant to microbial-host interactions.

647 Molecular and Cellular Proteomics 11 DOI: 10.1074/mcp.M111.015487.

648 Winkel KD, Tibballs J, Molenaar P, Lambert G, Coles P, Ross-Smith M, Wiltshire C,

649 Fenner PJ, Gershwin LA, Hawdon GM, Wright CE, Angus JA. 2005. Cardiovascular

650 actions of the venom from the Irukandji (*Carukia barnesi*) jellyfish: Effects in human,

651 rat and guinea-pig tissues *in vitro* and in pigs *in vivo*. Clinical and Experimental

652 Pharmacology and Physiology 32:777–788 DOI: 10.1111/j.1440-1681.2005.04258.x.

653

654 **Figure legends**

655 **Figure 1:** A) *Chrysaora lactea*, B) *Tamoya haplonema* and C) *Chiropsalmus*

656 *quadrumanus*. Medusa adult stages of Cnidaria from which the venom proteomes of

657 isolated nematocysts were acquired for this study. (Photos courtesy of Dr Alvaro

658 Migotto, Centro de Biologia Marinha, Universidade de São Paulo

659 São Sebastião, Brasil).

660 **Figure 2. Comparison of Cnidarian venom composition.** Venn diagram showing the

661 number of putative toxin protein families shared among the soluble nematocyst

662 proteomes of the four classes of cnidarians studied (Note that the protein families

663 marked with an asterisk are described here for the first time).

664 **Figure 3. Recruitment patterns of putative toxin protein families into Cnidaria**

665 **venom, based on a established cnidarian phylogenies** (Marques & Collins, 2004;

666 Collins *et al*., 2006). Solid black rectangles represent recruitment events. Dotted

667 rectangles represent absence of toxin families. White rectangles represent multiple

668 recruitments of toxin families. The numbers above of the lines represents the toxin

669 families: 1. actinoporins; 2. complement C3; 3. conotoxins O; 4. Conotoxins T; 5.

670 CRISP; 6. ficolin lectin; 7. flavin monoamine oxidase; 8. Jellyfish toxin; 9. kunitz-type;

671 10. latrotoxin; 11. MAC-PF; 12. metalloproteinase; 13. natriuretic peptide; 14. peptidase

672 S1; 15. phospholipase A2; 16. phospholipase B; 17. phospholipase D; 18. ShK-like

673 potassium channel; 19. snaclec; 20. snake three finger; 21. Sea anemone sodium

674 channel modulator; 22.TCTP; 23. glycosyl hydrolase 56*; 24. huwentoxin-1*; 25.

675 latarcin*; 26. lipase*; 27. CS αβ potassium channel blocker*; 28. CS αβ sodium channel

676 inhibitor*. The proteins families marked with asterisk (*) have never previously been

677 recorded in Cnidaria.

Table 1: **Predicted venom proteomes of potential toxins isolated from nematocysts**. A) *Chiropsalmus quadrumanus*, B) *Tamoya haplonema*

and C) *Chrysaora lactea*. Peptide fragments used for putative toxin annotation are given with validated spectra in Figures S1-S3.

Toxin with closest homology	Predicted toxin protein family	Uniprot accession number	Example of animal species with closest homology
A) *Chiropsalmus quadrumanus*			
Alpha-latroinsectotoxin-Lt1a	Latrotoxin	Q02989	*Latrodectus tredecimguttatus* (European black widow spider)
Conotoxin Bu2	Conotoxin O1	P0CY61	*Conus bullatus* (Bubble cone snail)
Echotoxin-2	Actinoporin	Q76CA2	*Monoplex parthenopeus* (Giant triton sea snail)
Hainantoxin-XVIII-5	Putative ion channel inhibitor	D2Y2N9	*Haplopelma hainanum* (Chinese bird spider)
Neurotoxin LmNaTx1	CS αβ sodium channel inhibitor	D9U297	*Lychas mucronatus* (Chinese swimming scorpion)
Toxin CfTX-2	Jellyfish toxin	A7L036	*Chironex fleckeri* (Sea wasp)
B) *Tamoya haplonema*			
Alpha-latroinsectotoxin-Lt1a	Latrotoxin	Q02989	*Latrodectus tredecimguttatus* (European black widow spider)
Conotoxin Lt5.9	Conotoxin T	Q1A3Q7	*Conus litteratus* (Lettered cone snail)
DELTA-alicitoxin-Pse2b	MACPF	P58912	*Phyllodiscus semoni* (Wasp sea anemone)
Disintegrin acostatin-alpha	Disintegrin	Q805F7	*Agkistrodon contortrix contortrix* (northern copperhead pit viper)
Echotoxin-2	Actinoporin	Q76CA2	*Monoplex parthenopeus* (Giant triton sea snail)
Equinatoxin-3	Actinoporin	P0C1H2	*Actinia equina* (Beadlet sea anemone)

Im-conomarphin	Conotoxin A	P0CH39	*Conus imperialis* (Imperial cone snail)
Maximins 3/H2	Bombinin	P83082	*Bombina maxima* (Yunnan firebelly toad)
Phospholipase A2 3	Phospholipase A2	P21792	*Micrurus nigrocinctus* (Central American coral snake)
Phospholipase D LlSicTox-alphaIII2	Arthropod phospholipase D	Q1KY79	*Loxosceles laeta* (Chilean recluse spider)
Potassium channel toxin alpha-KTx Tx773	CS αβ potassium channel blocker	B8XH45	*Buthus occitanus israelis* (Common yellow scorpion)
Potassium channel toxin TdiKIK	Long chain scorpion toxin	Q0GY43	*Tityus discrepans* (Venezuelan scorpion)
Snake venom metalloproteinase aculysin-1	Venom metalloproteinase (M12B)	Q9W7S2	*Deinagkistrodon acutus* (Sharp-nosed pit viper)
U5-ctenitoxin-Co1a	Spider toxin Tx2	P85276	*Ctenus ornatus* (Brazilian spider)
Venom allergen 5	CRISP	A9QQ26	*Lycosa singoriensis* (Chinese wolf spider)
Venom carboxylesterase-6	Lipase	B2D0J5	*Apis mellifera* (European honey bee)
Venom nerve growth factor 1	NGF-beta	Q2XXL6	*Azemiops feae* (Black-headed Burmese viper)

C) *Chrysaora lactea*

CfTX-2	Jellyfish toxin	A7L036	*Chironex fleckeri* (Sea wasp)
Cathelicidin-related peptide Na_CRAMP	Cathelicidin	B6S2X0	*Naja atra* (Chinese cobra)
Conotoxin Bu2	Conotoxin O1	P0CY61	*Conus bullatus* (Bubble cone snail)
Cysteine-rich venom protein LIO1	CRISP	Q2XXQ0	*Erythrolamprus poecilogyrus* (Water snake)
L-amino-acid oxidase	Flavin monoamine oxidase	P0DI84	*Vipera ammodytes* (Sand viper)
M-zodatoxin-Lt4a	Latarcin	Q1ELU5	*Lachesana tarabaevi* (Ant spider)
Snake venom serine protease KN2	Peptidase S1	Q71QJ0	*Trimeresurus stejnegeri* (Chinese green tree viper)

U16-lycotoxin-Ls1a	U16-lycotoxin	B6DD52	*Lycosa singoriensis* (Chinese wolf spider)
Venom peptide Ocy2	Not assigned	P86107	*Opisthacanthus cayaporum* (South American scorpion)

Hydrozoa

Cubozoa

Anthozoa

Scyphozoa

0

0

1 - - - → MAC-PF

1

1

TCTP ◄- - -

1

0

1

3

→ JellyFish toxin

1 - - - → Ficolin lectin

ShK-like potassium channel
Sea anemone sodium channel
modulator
Phospholipase B

0

9

3

Complement C3
Huwentoxin-1*
Natriuretic peptide

1

5

Actinoporin

2

Conotoxins T

Kunitz-type
Latarcin*

Flavin monoamine oxidase
Snaclec
Snake three finger
Glycosyl hydrolase 56*
Peptidase S1

Conotoxins O
CRISP
Latrotoxin
Metalloproteinase
Phospholipase A2
Phospholipase D
Lipase*
CS αβ potassium channel blocker*
CS αβ sodium channel inhibitor*

- Early diverging metazoans offer a phylogenetic anchor to study evolution of the venom trait.

- Venom proteomes of the scyphozoan *Chrysaora lactea* and two cubozoans *Tamoya haplonema* and *Chiropsalmus quadrumanus* are presented.

- Toxin recruitment and retention patterns do not always correlate with accepted phylogeny.

- Factors that drive toxin diversification independent of phylogeny merit closer scrutiny.